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Long-Term Survival of Enteric Microorganisms in Frozen Wastewater

Louise V. Parker and C. James Martel

October 2002

Abstract: Microorganisms that are found in domestic wastewater and that can cause illness in humans include bacteria, viruses, protozoan cysts, and helminth ova. This literature review attempts to determine whether organisms contained in a frozen sewage bulb in the Antarctic ice would survive for decades. This review briefly examines the structural differences between these organisms; examines the susceptibility of these organisms to chilling, freezing, thawing, and frozen storage, and the effect these processes have on the structural components of the organisms; and compares findings from field studies, including some archeo-logical studies, on the ability of these organisms to withstand natural cold environments.

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Prepared for
NATIONAL SCIENCE FOUNDATION

PREFACE

This report was prepared by Louise V. Parker, Research Physical Scientist, and C. James Martel, Environmental Engineer, Applied Research Division, U.S. Army Engineer Research and Development Center (ERDC), Cold Regions Research and Engineering Laboratory (CRREL), Hanover, New Hampshire.

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Contents

ILLUSTRATIONS

TABLES

Long-Term Survival of Enteric Microorganisms in Frozen Wastewater

LOUISE V. PARKER AND C. JAMES MARTEL

1 INTRODUCTION

Typically, the microorganisms found in domestic wastewater come from the human gastro-intestinal tract and are referred to as enteric microorganisms. They include bacteria, viruses, protozoa (in cyst form), and helminths or parasitic worms (primarily in the egg or ova stage). Although many of these microorganisms cause no problem for their host, others cause gastro-intestinal illnesses characterized by diarrhea and abdominal cramps, which may be accompanied by vomiting and fever. Occasionally, these microorganisms can cause more serious illnesses, which will be discussed later.

The South Pole Station uses approximately 12,800 L (3,400 gallons) of water per day in the austral summer and 3,800 L (1,000 gallons) per day in the austral winter (NSF 1997). Most of this water becomes wastewater since there is little or no consumptive use (e.g., lawn watering, industrial operations) at the station. This wastewater is collected and discharged into an old water well or sewage bulb in the snowpack above the glacial ice. It is typically 37 meters deep and 37 meters in diameter (Reed and Sletten 1989). Several sewage bulbs are in use: one located several hundred meters from the station, and some smaller bulbs located beneath the summer camp. Figure 1 shows a sketch of a similar sewage bulb on the Greenland ice cap.

From time to time, there are reports of microorganisms that have survived for long periods at subfreezing temperatures being recovered from a remote polar location. As an example, two teams of scientists (Karl et al. 1999, Priscu et al. 1999) recently found viable microorganisms in the accreted ice above one of the subglacial lakes in Antarctica. However, it is not clear whether the various types of enteric microorganisms contained in sewage and discharged into the polar ice cap would survive. Any surviving microorganisms could potentially present either an environmental threat or a health threat to humans if portions of the ice

cap were to reach the ice edge, break off, and melt, or if contaminated meltwater reached a sensitive area.

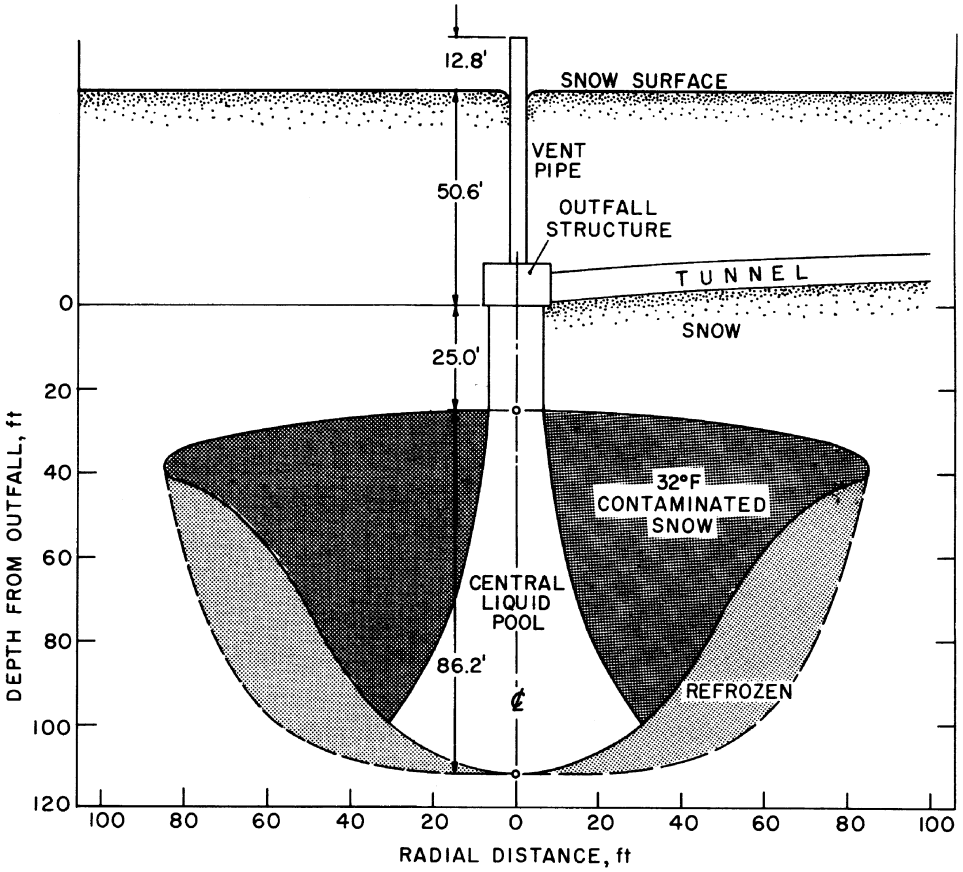

Figure 1. Diagram of sewage bulb in Greenland ice cap. (From Reed and Sletten 1989.)

Previous research at CRREL focused on the survival of several types of pathogens in frozen sludge (Martel 1984, Sanin et al. 1994). These studies found that freezing negatively affected most types of pathogens, but many survived, especially helminth ova (*Ascaris suum*). However, the maximum freezing time in these studies was only 28 days.

We have also studied the survival of wastewater bacteria during snowmaking (Parker et al. 2000). Although we were unable to recover any coliform bacteria following snowmaking, other bacteria, including fecal streptococci, survived.

The focus of this literature review is to look more closely at the effects of low temperature, freezing, and thawing on various types of sewage-borne microorganisms, especially those of public health concern, and to explore their long-term survival (i.e., decades and longer) in a frozen environment. The cellular structure of sewage-borne organisms varies considerably, and because cell structure affects an organism's susceptibility to chilling and freezing, we will briefly examine cellular structure of the various sewage-borne microorganisms.

Cell structure

Bacteria and protozoa are single-celled, or unicellular, organisms and almost all are microscopic in size. In contrast, most multicellular organisms can be seen with the naked eye and, in the mature state, have a degree of structural complexity. In higher animals, multicellularity gives rise to distinct tissue regions, and different tissues can form specialized local structures known as organs.

All cells contain genetic information (DNA) that is surrounded by cytoplasm (a fluid that contains the RNA and protein of the cell). This is separated from the external environment by a membrane known as the cytoplasmic or cell membrane, which is composed of proteins and lipids. In some cells, this membrane is the only bounding structure. For other types of cells, the cytoplasmic membrane is surrounded by a much thicker and sturdier structure with a different composition known as the cell wall. These organisms can be differentiated by these fundamental differences in cell structure. The eucaryotic cell is more highly differentiated and is the unit of structure for protozoa and animals, including parasitic worms. The procaryotic cell is less differentiated and is the unit of structure for bacteria.

Eucaryotic cells are structurally complex with a distinct nucleus that is surrounded by a nuclear membrane. The genetic information of eucaryotes is contained in a number of chromosomes. Eucaryotes also contain a number of membrane-bounded organelles that carry out specific functions of the cell (Fig. 2). These organelles can include mitochondria (which are the sites of cellular respiration), the endoplasmic reticulum (which functions as a surface upon which ribosomes and other components of the protein-synthesizing machinery are located), lysosomes (membrane-bound bodies that contain hydrolytic and digestive enzymes), vacuoles (liquid-filled bodies with various functions including food digestion, osmotic regulation, and excretion of waste products), and the golgi apparatus (which is involved in producing vacuoles that are involved in transport of various cellular products).

There are considerable differences with respect to the nature of the surface layer of these cells. For all eucaryotes, the cell membrane contains sterols. For

animals and many protozoa some cells, the cell membrane is the only bounding structure. Although protozoa typically do not have cell walls, they do have a surface layer that provides an element of rigidity. For example, (non-photosynthetic) flagellate and ciliate protozoa have a thin flexible outer layer composed of protein termed a pellicle. Sexual reproduction is also considerably more complex for eucaryotes than for procaryotes.

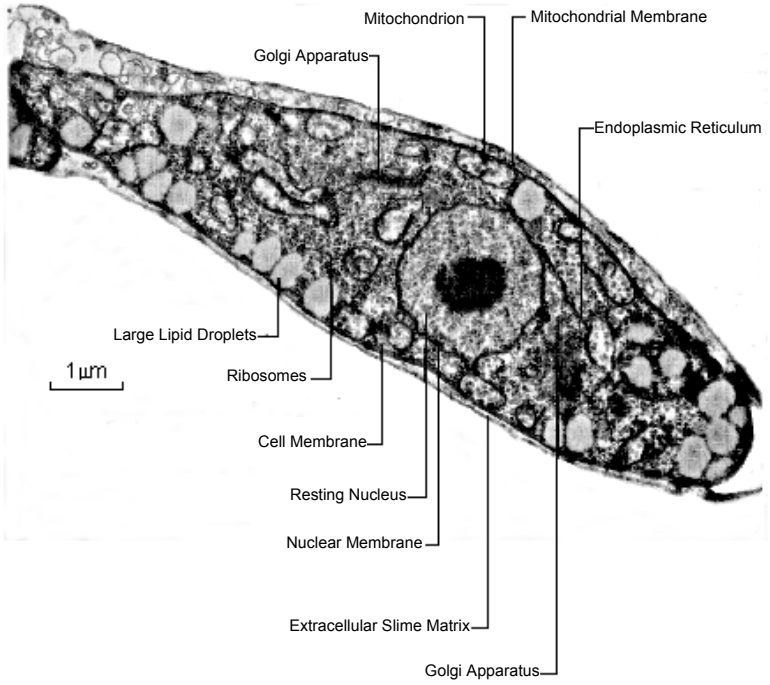

Figure 2. Electron micrograph of non-photosynthetic eucaryotic protist. (After Brock 1970.)

The genetic material of procaryotes is not contained in a nucleus but rather is a single exposed strand of DNA in the cytoplasm. These organisms also lack membrane-bound organelles and sterols in the cell membrane (Fig. 3). However, virtually all procaryotes have cells enclosed by rigid walls that are more chemically complex than the cell membranes of eucaryotes. The cell wall of procaryotes contains a murein layer, which consists of two sugar derivatives (amino-sugars) and several amino acids. Bacterial cell walls are often very complex in structure and can contain other polymeric constituents.

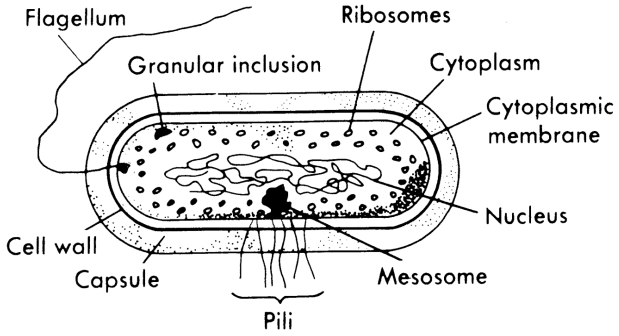

Figure 3. Schematic of a procaryotic cell. (From Davis 1973.)

Viruses are a non-cellular form of life and thus are neither procaryotes nor eucaryotes. Typically, viruses are sub-microscopic particles (i.e., they must be seen with an electron microscope), although some are as large as the smallest bacteria. All are obligate intracellular parasites. Plants, animals, and even bacteria are subject to infection by viruses. Viruses are structurally very simple and consist primarily of a nucleic acid, either DNA or RNA, contained in a protein or lipo-protein coat. They are not capable of carrying out metabolic functions on their own and posses no enzyme systems for energy production. They cannot grow or undergo binary fission on their own but rather are reproduced from their nucleic acid by the host cell.

2 GENERAL EFFECTS OF FREEZING ON LIVING CELLS

Mazur (1966, 1984) has studied the effects of chilling and freezing on several types of cells, including both eucaryotes and procaryotes. The following discussion on the general effects of freezing on living cells is taken from Mazur (1984). Although lowered temperature can cause cell injury in some cells under certain circumstances, low-temperature injury in most cases is associated with ice formation. The primary challenge to cells that have been frozen is not their ability to endure storage at very low temperatures but rather the lethality of an intermediate zone of temperature (~ –15°C to –60°C) that a cell must traverse during freezing and warming.

Extremely low temperatures (–130°C and lower) do not have much negative effect on cells. The only reactions that can occur in frozen aqueous systems at –196°C (the storage temperature in liquid nitrogen) are photophysical events (if light is present), such as the formation of free radicals, and the production of breaks in macromolecules as a direct result of hits by background ionizing radiation or cosmic rays. With time, these direct ionizations can produce enough breaks and/or other damage in DNA to become deleterious after rewarming to physiological temperatures, especially since no enzymatic repair can occur at these very low temperatures. As an example, it is estimated that at –196°C, it would take an estimated 2,000 to 4,000 years for ionizing radiation to kill 63% of cultured mammalian cells. Also, liquid water does not exist below –130°C. The only physical states for water that exist at that temperature are crystalline or glassy, and in both cases diffusion would be insignificant in less than geological time. Therefore, stability for centuries or millennia requires temperatures below –130°C, where the biological clock is essentially stopped. Many cells stored above –80°C are not stable, probably because traces of unfrozen solution exist.

At temperatures down to approximately –5°C, cells and their surrounding medium remain unfrozen because of supercooling and the depression of the freezing point by solutes. Between –5°C and approximately –15°C, ice forms in the external medium but the cell contents remain unfrozen and supercooled, because the plasma membrane blocks the growth of ice crystals in the cytoplasm. The supercooled water in the cells has a higher chemical potential than that of water in the partly frozen solution outside the cell, and in response to the difference in potential, water flows out of the cell and freezes externally. At approximately –40°C, homogenous ice nucleation occurs (i.e., water freezes spontaneously in the absence of ice nucleators).

The subsequent physical events in the cell depend upon the cooling velocity. Generally, survival of cells is optimum at some intermediate cooling rate, the value of which depends on the particular cell. If cooling is sufficiently slow, the cell is able to lose water rapidly enough by exosomosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. The result is that the cell dehydrates and does not freeze intracellularly. However, if the cell is cooled too rapidly, it is not able to lose water fast enough to maintain equilibrium and becomes increasingly supercooled and eventually attains equilibrium by freezing intracellularly. Cooling rates that cause intracellular freezing also cause extensive cell death. There is evidence that intracellular freezing is the cause of death and not a consequence of it.

Cells that have been cooled rapidly enough to freeze intracellularly are much more sensitive to slow warming than to rapid warming. At extremely rapid cooling rates, the crystals become so small that they cannot be seen at even the ultramicroscopic level. There is a tendency during warming for these ultra-small crystals to aggregate and form larger crystals, a process that is referred to as recrystallization. The general consensus is that slow warming is harmful to rapidly frozen cells because it allows time for recrystallization to occur.

Slow freezing (i.e., at a rate slower than the optimum rate) also causes injury. This is because the only ice that forms under these conditions is extracellular. Therefore, any observed injury is either a consequence of the direct action of that ice, of alterations in the proportion of ice to extracellular solution, or of changes in the composition of the external solution brought about by the conversion of water into ice. Solute concentrations inside and outside the cell can reach levels that cause denaturation of proteins and breakdown of membranes. Photomicrographs of cells subjected to hyperosmotic solutions during slow freezing versus cells exposed to the same solution without freezing suggest that the physical forces due to freezing are primarily responsible for cell injury.

For cells that have been cooled more slowly than the optimum rate, survival is considerably higher with slow warming than with rapid. One reason for this is that slowly frozen cells exposed to rapid thawing may be exposed to osmotic shock. There is evidence that additional solutes may be driven into cells during slow freezing, a process referred to as solute loading. If so, there may be insufficient time for the excess solute to diffuse back out when thawing is rapid and the cells swell and lyse as the medium is abruptly diluted by the melting extracellular ice.

The properties of the cell membrane help determine a cell's response to freezing. The structure of the surface membrane may allow cells to supercool

and thus determines their ice-nucleation temperature. The nucleation temperature and the permeability of membranes to water are the chief determinants of whether cells will equilibrate by dehydration or intracellular freezing. Furthermore, surface and internal membranes seem to be the chief targets of injury with both slow and rapid freezing.

In a sewage bulb, presumably microorganisms would be subjected to a slow freezing rate (i.e., less than the optimum rate) and to a final temperature of approximately –51°C (–60°F), where long-term survival is more probable than around freezing (0°C). We will now examine the survival of each type of microorganism in more depth, starting with bacteria.

3 BACTERIA

General information

Bacteria are typically microscopic in size, although some are submicroscopic and require an electron microscope to be seen. Typical shapes and sizes of bacteria include cocci (spheres) (1- to 3-μ diameter), straight or curved rods (cylindrical shapes) (0.3- to 1.5-μ width, 1- to 10-μ length), helically shaped spirals (up to 50 μ), and filamentous forms (100 μ or longer) (Fig. 4). Bacteria may be motile by means of flagella or by an axial filament for spiral shapes. Others contain endospores that are resistant to high temperatures and dessication.

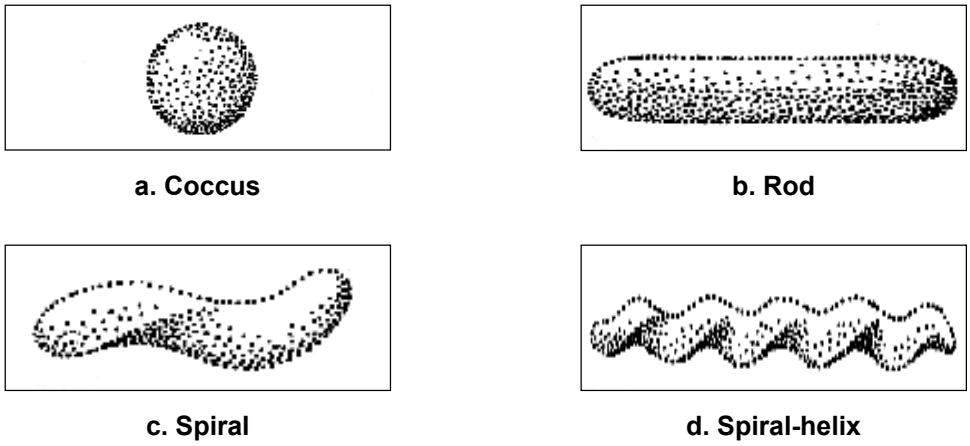

Figure 4. Representative bacterial shapes. (From Brock 1970.)

Bacteria have been classified using aspects of their cell morphology (their size, shape, ability to be mobile, presence of flagella, and ability to form spores), their ability to utilize and degrade various carbon sources, and their genetic and biochemical makeup. One useful mechanism for differentiating bacteria is their reaction to a series of dyes or stains, especially the Gram Stain. Gram-positive bacteria have a much thicker cell wall and thus retain the original purple stain and are not decolorized by an organic solvent solution. Gram-negative bacteria have a thinner cell wall and thus are readily decolorized by the solvent mixture; they are counter-stained with a red dye so they can be observed under the microscope. However, gram-negative bacteria have another protective layer, a cell envelope, which is a layer of lipopolysaccharide and protein.

Numbers and types found in sewage and feces

Sewage bacteria of concern include some species of *Shigella, Salmonella, Leptospira, Vibrio*, *Yersinia, Bacteroides,* and *Campylobacter*, enterotoxin-producing strains of *Bacteroides fragilis*, and certain strains of *Escherichia coli (E. coli)*. Illnesses other than gastroenteritis that these organisms can cause include acute infections of the kidneys, liver, and central nervous system, and even death. Minimal infective doses range from 10 to 100 cells for *Shigella* spp. and from 10^6 to 10^9 cells for *E. coli* (Bitton 1999, Carodona 2000) (Table 1).

Table 1. Minimal infective doses for some microorganisms and concentrations commonly found in raw wastewater.

Organism	Minimal infective dose[a]	Minimal infective dose[b]	Concentration MPN/100 mL[b]
Bacteria			
Salmonella spp.	10^4–10^7		10^2–10^4
Shigella spp.	10^1–10^2	10–20	1–10^2
Escherichia coli	10^6–10^8		
Fecal coliforms		10^6–10^{10}	10^6–10^8
Vibrio cholerae	10^3		
Campylobacter jejuni	10^2–10^6		
Viruses (PFU)			
Hepatitis A virus	1–10		
Enteric virus		1–10	10^3–10^4
Protozoa (cysts)			
Giardia lamblia	10^1–10^2	< 20	10^3–10^4
Cryptosporidium	10^1	1–10	10–10^3
Entamoeba coli	10^1		
Helminths (eggs)			
Ascaris	1–10		

[a] From Bitton (1999)

[b] From Carodona (2000)

PFU = Plaque Forming Units

Feces can contain up to 10^{12} bacteria per gram, and infected individuals can excrete up to 10^9 *Shigella* per gram feces (Bitton 1999). Reported concentrations of various species of bacteria in raw wastewater have ranged from a few to 8,000 *Salmonella*/100 mL, from 10 to 10^4 *Vibrio cholerae*/100 mL, and from 10^4 to 10^5 *Bacteroides fragilis*/mL (Bitton 1999). Carodona (2000) reported concentrations of 1 to 10^2 *Shigella* spp./100 mL and slightly higher concentrations of *Salmonella* spp. (10^2 to 10^4/100 mL) (Table 1).

Studies have shown that bacterial particulates released to aqueous environments from sewage attach to surfaces such as sediment particles and to each other, and other enteric bacteria have a tendency to partition into/onto lipoidal particulates (Grimes et al. 1986). This greatly affects subsequent mobility in a water body and may aid in their survival.

Effects of chilling, freezing, low-temperature storage, and warming on bacteria

Bacteria can be injured or die as a result of rapid chilling without freezing (cold shock), freezing, storage at low or sub-zero temperatures, and subsequent warming. Studies have shown that cold shock can damage the cytoplasmic (inner) membrane and DNA of bacteria and can damage the outer membrane of gram-negative bacteria (MacLeod and Calcott 1976, Mackey 1984). In addition to this damage, freezing and thawing can also damage the cell wall (MacLeod and Calcott 1976, Mackey 1984, Ray 1989). When the cytoplasmic membrane is damaged, low-molecular weight material (such as potassium and magnesium cations, inorganic phosphate, and amino acids) are lost from the cell, and there is an increased penetrability of small molecular weight compounds (such as toxic metals) into the cell (Ray and Speck 1973, MacLeod and Calcott 1976). Researchers have attributed death and injury to one or both of these processes. However, depending upon the species and the surrounding medium, many of the cells injured by these processes can undergo some level of self-repair.

Both gram-positive and gram-negative bacteria can be affected by cold shock (MacLeod and Calcott 1976). Factors that affect the sensitivity of bacteria to cold shock include age (cold shock usually occurs in cells harvested in the exponential growth but not stationary phase), composition of the medium in which cells are chilled (divalent cations substantially protect against the effect of chilling and have been shown to mediate recovery), cell number (loss of viability is greater the smaller the cell population), rate of cooling, and temperature range over which cooling takes place.

For bacteria that have been frozen and thawed, both the rates of cooling and warming affect survival and produce different kinds of damage (MacLeod and

Calcott 1976). For many species, the optimum cooling rates are between 6 and 11°C per minute (Mazur 1966, MacLeod and Calcott 1976, Mackey 1984). Damage also varies depending on the chemical composition of the freezing medium, especially the presence of NaCl (MacLeod and Calcott 1976). The type and strain of organism, its phase of growth when frozen, and the temperature and duration of frozen storage are also important (Mackey 1984). The initial number of bacteria also affects survival, with high concentrations having a protective effect (Mazur 1966). Resistance of bacteria to freezing varies widely; cell shape and differences in membrane fatty acids and proteins have been found to affect cryosensitivity (Mackey 1984).

During rapid cooling, permeability changes in the cell membrane are caused by a phase transition in the membrane lipids from a liquid crystalline to a gel state (MacLeod and Calcott 1976). Slow cooling allows a lateral phase separation of the lipids and proteins of the membrane, whereas rapid cooling "fixes" these components in a random, disordered state resulting in membrane leakiness (Mackey 1984).

Although considerable strain variation exists, vegetative cells of cocci are resistant to freezing and frozen storage, gram-negative bacteria are generally less resistant than gram-positive bacteria, and spores are very resistant to freezing (Speck and Ray 1973, 1977; Kraft 1992). Studies by Mackey (1984) demonstrated this for several types of bacteria stored at –20°C and by McCarron (1965) for several types of bacteria stored at –2°C and –20°C. In particular, fecal coliforms and *E. coli* are more susceptible to freezing than many other species of bacteria, especially gram-positive fecal streptococci (McCarron 1965, Mackey 1984, Sanin et al. 1994, Parker et al. 2000).

Several studies have shown that in addition to the death of cells on initial freezing, there is usually further death during frozen storage. Usually, death occurs rapidly in the early stages followed by a slowing of the rate until, in the later stages, numbers remain almost constant, with greater survival at lower temperatures (McCarron 1965, Ray and Speck 1973, Mackey 1984). As would be expected based on previous discussion, death rates are low or zero when storage is at temperatures of –70°C or below, whereas the survival of most species decreases with time at temperatures between 0°C and –60°C (Mazur 1966, Ray and Speck 1973). The rate of the decrease in survival also depends on the species, the nature of the freezing medium, and in some cases the cell concentration (Mazur 1966, MacLeod and Calcott 1976). Death is presumed to be mainly due to continued exposure to concentrated solutes (Mackey 1984). Organisms in logarithmic growth phase are not as resistant as those in stationary phase (Kraft 1992).

Repeated freezing and thawing has also been shown to increase bacterial injury for a number of species (Ray and Speck 1973). Nelson and Parkinson (1978) examined the effects of various freeze–thaw cycles (to –22°C) on bacteria isolated from arctic soils. They found that gram-negative *Pseudomonas* species were the most susceptible; gram-positive *Arthrobacter* species were highly resistant; and the gram-positive, spore-forming *Bacillus* species were intermediate in their resistance. During a global period of warming, Antarctic sewage microorganisms could undergo several freeze–thaw cycles.

Cold adaptation

Long-term exposure to cold can induce physiological changes that permit growth below the normal lower growth limit of bacteria (Ray and Speck 1973, Smith et al. 1994). As an example, prolonged exposure (54 days) of several species of human enteric bacteria to a marine polar environment (at –1.8°C) shifted the optimal and permissive growth temperatures down to temperatures as low as –1.8°C (Smith et al. 1994).

Russian studies have shown that the long duration of temperatures of –45°C in Siberia extended persistence of *Shigella* in feces, soil, and frozen river water (Geldreich 1972). Researchers have also reported that freezing, thawing, or prolonged frozen storage does not result in loss of virulence for many pathogenic bacteria (Geldreich 1972, Ray and Speck 1973, Speck and Ray 1977). However, Geldreich (1972) noted that these pathogens did undergo some modification of biochemical characteristics during their exposure to subzero temperatures. These changes can make detection by conventional laboratory procedures difficult and will be discussed shortly.

Exposure to cold or freezing can also be associated with adaptation by biosynthesis of antifreeze proteins, membrane stabilizers, or cold shock proteins (McGrath et al. 1994). Also, metabolic changes can alter membrane compositions, and adaptation of protein structures are often found in cold-adapted organisms (McGrath et al. 1994, Smith et al. 1994).

McGrath et al. (1994) conducted studies on bacteria isolated from ancient Siberian permafrost soil. They found these organisms survived in the frozen state (–10°C to –13°C) by one or more of the following means. Many produced gelatinous sheets, which may act as a water reservoir in the dehydrated state. Some microorganisms also produced thickened walls, which may protect against mechanical damage by extracellular ice. Also, intracellular membranes were found in some of these organisms. Whole cell fatty acid analysis revealed an increase in mono-unsaturated fatty acid content and a decrease in saturated fatty acid content of cells grown at 4°C relative to those grown at 15°C. Presumably,

changes in the fatty acid composition would affect the membranes, making them more stable, and possibly affect cell activity in the frozen state by altering membrane permeability (McGrath et al. 1994).

Differential scanning calorimetry revealed that there was no evidence of intracellular ice formation in the Siberian permafrost microorganisms, even for cells frozen to temperatures as low as −150°C (McGrath et al. 1994). This demonstrates that freezing in the environment is probably too slow for destructive intracellular ice formation to occur, and intracellular solutes play an important role in the freezing process (McGrath et al. 1994). These results suggested that these organisms contain a highly concentrated but unfrozen intracellular solution, which may allow metabolism while the microorganism is encased in the extracellular ice (McGrath et al. 1994). The increased level of intracellular solutes would depress the freezing point and reduce the extent of shrinkage that typically occurs. Intracellular cryoprotective solutes at concentrations of 1 to 2 M can depress nucleation temperatures from –10°C down to temperatures of –40°C to –60°C (McGrath et al. 1994).

Many Antarctic bacteria also have thickened walls and/or mucilaginous sheaths and employ a strategy of polyol accumulation and synthesis of mucilaginous sheaths, presumably for the purpose of water budgeting and polyol retention (McGrath et al. 1994). It has also been proposed that these sheaths play a role in preventing the adhesion of ice crystals to the cell walls as well as lowering the freezing point of the intracellular microenvironment (McGrath et al. 1994). This type of adaptation would certainly improve the survival of frozen sewage-borne bacteria.

Stasis, cell injury, and cell death

Many bacteria that are believed to have been killed by freezing, frozen storage, or thawing (especially gram-negative bacteria) are actually only injured as a result of sublethal physiological and/or structural change. A bacterium is termed injured if it can form colonies on nonselective medium but not on medium that contain selective agents to which the organism is normally resistant. Because many methods used to enumerate viable pathogenic bacteria utilize selective media, these methods can grossly underestimate the number of these organisms present. This would be true for bacteria that have been subjected to freezing, thawing, chilling, etc. To recover injured bacteria, a pre-enrichment step is recommended prior to using a selective medium (Ray 1989).

Also, many human enteric bacteria exposed to a marine or estuarine environment enter an altered physiological state termed viable but nonculturable (Xu et al. 1982, Roszak et al. 1984, Colwell et al. 1985, Grimes et al. 1986, Smith et al.

1994). This is commonly defined as the inability to form colonies on a given solid medium while remaining physiologically active. Physiological activity can be determined by a number of methods including direct viable counts (DVCs) or measuring respiratory activity. Smith et al. (1994) found 99% of *E. coli*, *Salmonella typhimurium,* and *Yersinia enterocolitica* entered a viable but unculturable state when exposed to a marine polar environment (–1.8°C) for 54 days. They also found that it was nutrient availability rather than temperature that limited bacterial activity in this cold environment.

Garcia-Lara et al. (1991) have compared mortality rates of fecal bacteria in seawater using direct microscopic counts, plate counts on selective and non-selective media, and a method that follows the disappearance of radioactively labeled DNA in labeled bacteria. For *E. coli*, the rates of disappearance in sterile seawater were ~1.5 times greater using a selective medium than using a non-selective medium. The rates of disappearance using direct microscopic observation and the labeled-DNA method were more than two orders of magnitude less than the rate observed using culture on a selective medium. Similar differences were seen for *S. typhimurium* but these differences were considerably less pronounced for *Streptococcus faecium*.

Survival in cold environments—Field studies

A number of researchers have examined the persistence of microorganisms at the poles, primarily in permafrost but also in snow and ice. As early as 1911 and 1912 Russian scientists reported recovering viable organisms from permafrost soils (Gilichinsky and Wagener 1995). In 1961, Becker and Volkmann recovered eight bacteria from four different genera from permafrost cores taken in Fairbanks, Alaska (Gilichinsky and Wagener 1995). Preliminary studies by Cameron and Morelli (1974) found viable organisms from drill cores (permafrost) from the ancient Ross Island and Taylor Valley in Antarctica. Among the organisms isolated were rod-shaped bacteria-like organisms that were motile by means of flagella, yeast-like and streptomycete-like microorganisms, and pleomorphic, rod-shaped microorganisms resembling coryneform bacteria (a predominant group found in pristine Antarctica). The authors believed that these samples were recovered from the Pleistocene period (8,000 to 1.8 million years ago).

Although earlier accounts were somewhat suspect because of the drilling methods used, more recent studies have used specially designed samplers. Using newer sampling methods, Russian scientists have found high numbers of viable microorganisms (up to 10^8 cells/g) in ancient (3- to 5-million-year-old) Siberian permafrost soils (McGrath et al. 1994, Gilichinsky and Wagener 1995). The temperature of these soils reportedly ranged from –10°C to –13°C. McGrath et

al. (1994) noted that these densities were comparable to concentrations found in common soils at more moderate temperatures. These organisms were widely divergent, both morphologically and physiologically (Gilichinsky and Wagener 1995). Organisms included coccoid, coryne-like, nocardia-like, and rod-like gram-positive and gram-negative bacteria; actinomycetes; fungi; and yeasts. These organisms were both aerobic and anaerobic. Anaerobes included sulfate reducing bacteria, *Clostridia* spp., actinomycetes, *Propionibacterium* spp., and methanogenic archaeobacteria. Most organisms were psychrotrophic (with optimum growth at 20°C), and 95% did not grow at temperatures higher than 30°C (which are typical of most human pathogens).

In permafrost, 2 to 7% of the water remains unfrozen and is available as thin films that envelop organic and mineral particles and bacteria (Gilichinsky and Wagener 1995). Unfrozen water functions as a cryoprotectant against cell damage by ice crystals and as a nutrient medium (Gilichinsky and Wagener 1995). In frozen soils, nutrients reach the cells and waste products are eliminated by diffusion through the narrow channels of unfrozen water (Rivkina et al. 2000). These processes are limited by the thickness of the unfrozen films of water, which in turn depends on temperature (Rivkina et al. 2000). The thickness of these films decrease (asymptotically) from approximately 15 nm at –1.5°C to 5 nm at –10°C (Rivkina et al. 2000). Ultimately, the slow buildup of diffusion gradients progressively slows and perhaps stops the movement of both nutrients and waste materials (Rivkina et al. 2000).

According to Gilichinsky and Wagener (1995), frozen sediments and soils are abundant in viable cells because of the unfrozen water, while in pure ice their quantity is small. They attributed the relative sterility of fossil ice, compared to permafrost, to (1) initially low number of microbial cells due to cell exclusion during freezing (although they noted that water which forms ice veins is rich in microorganisms); (2) cell damage by ice crystals formed inside the cells (although they noted that this result is probably equally probable in both fossil ice and sediments); (3) cell damage by ice crystals growing outside the cells (mechanical cell disruption); (4) cell degradation due to metabolism (biochemical death if there is no release of metabolic products).

However, researchers recently found viable cells in snow and in ice samples. Carpenter et al. (2000) found surface snow samples taken from the South Pole contained bacteria that were metabolically active at temperatures as low as –12°C to –17°C. Skidmore et al. (2000) found metabolically diverse microbes in debris-laden ice from a high glacier. Abyzov et al. (1998) recovered a diverse community of procaryotes and eucaryotes from ice samples from a subglacial lake in Antarctica (Lake Vostok) that were over 100,000 years old (110,000 to 240,000).

Concentrations ranged from 10^3 to 10^4 cells/mL of ice melt, and temperatures in the glacier were –53 to –55°C.

Bacteria that were found included cocci, diplococci, rods of various lengths (straight and curved), and oval cells. Actinomycetes were also frequently encountered. Eucaryotes included yeasts (budding or dividing cells) and fungi (mycelial fragments and conidia). Although they did not find spore-forming bacteria, they noted that more ancient horizons of the glacier contained primarily spore-forming bacteria. Throughout this frozen geologic record, they noted a correlation between dust particles in the atmosphere and bacterial concentrations. They correlated this with global temperature decrease, more arid lands, and a period of greater wind intensity.

The organisms that have been found in glaciers may have had more time to become cold-adapted prior to freezing than those normally encountered in frozen sewage. However, there have been some reports of the survival of sewage microorganisms in Antarctica. Nedwell et al. (1994) reported total counts of bacteria of 10^6 cells/gram in pony dung that had been frozen for more than 80 years in samples that were taken from the sites of the 1907 Shackleton expedition and the Scott expedition of 1910–1911. Presumptive coliform counts were as high as 10^5 cells/gram but almost no confirmed coliform bacteria were found. Because the authors did not appear to use a pre-enrichment step that would aide in recovering the injured bacteria, it is possible that the concentrations of coliforms were higher than reported. Nedwell et al. (1994) found that the major groups of survivors were spore-formers, either *Bacillus* spp. or sporing actinomycetes, but noted that previous work by Meyer et al. (1962, 1963) had also recovered non-spore-forming organisms, including encapsulated *Pseudomonas* spp.

Conclusions

There is sufficient evidence that many species of enteric bacteria, including human pathogens, will survive freezing and frozen storage at temperatures found in a sewage bulb in Antarctica (–51°C or –60°F) for decades and probably for centuries.

4 VIRUSES

General information

Viruses are typically submicroscopic and range in size from 20 to 500 nm. A virion (a mature infectious particle) consists of a nucleic acid core, either RNA or DNA that is either double stranded or single stranded, enclosed by a protein coat called a capsid. The capsid is an arrangement of a variable number of subunits known as capsomeres. Generally, capsid symmetry can be either helical (where the capsid is a cylinder with a helical structure) (Fig. 5) or polyhedral (where the capsid is an icosahedron) (Fig. 6).

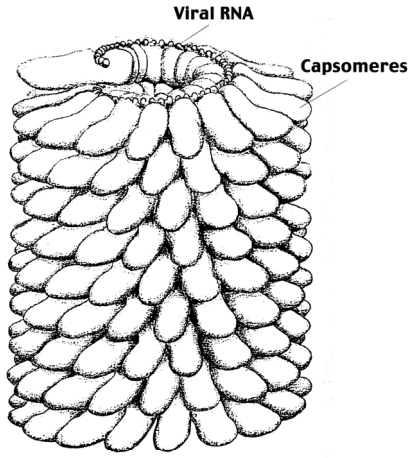

Figure 5. Tobacco mosaic virus. (From Goodheart 1969.)

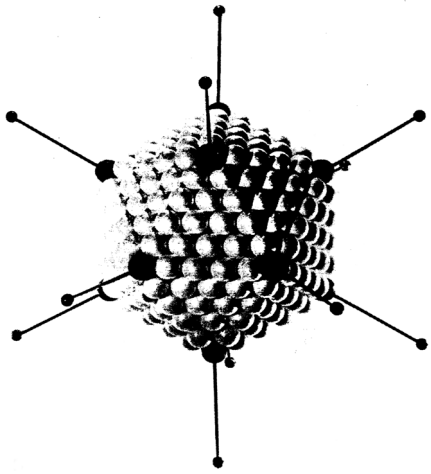

Figure 6. Human adenovirus type 5. (From Goodheart 1969.)

However, some viruses have a complex structure, such as the bullet-shaped Rhabdoviruses or the bacterial virus (bacteriophage) shown in Figure 7. The proteins in the capsomeres protect the nucleic acid from degradation, transcribe the nucleic acid (in the case of negative stranded genomes), and assist the virus in exposing the nucleic acid to the cell's biochemical machinery necessary for replication. Some viruses also contain carbohydrate or special enzymes not provided by the host cell. In some viruses, an envelope that contains lipoproteins or glycoproteins and lipids surrounds the capsid. Viruses that are devoid of an envelope are referred to as "naked virions."

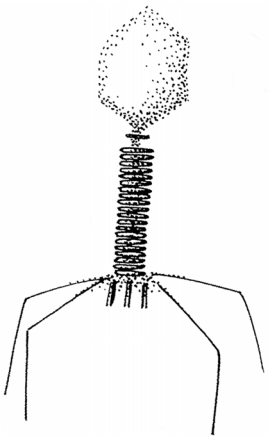

Figure 7. T_4 bacteriophage. (From Brock 1970.)

Typically, viruses are classified by viral size, capsid architecture, number of capsomeres in the capsid, the presence of an envelope around the capsid, the susceptibility of the virion to chemical solvents (which can damage lipid components of the envelope), the type of nucleic acid in the capsid, the site of replication of the nucleic acid (either in the cytoplasm or nucleus of the host cell), and the host for the virus (e.g., bacteria, animal, plant) (Bitton 1980).

Among the animal viruses, there are five major groups of DNA viruses (Table 2) and eight major groups of RNA viruses (Table 3). Enteric viruses of concern to humans include picornaviruses* (including polio, Coxsackie, echoviruses, and infectious hepatitis [type A] viruses), caliciviruses (including hepatitis E), reoviruses, adenoviruses, rotaviruses, astroviruses, parvoviruses, and caliciviruses (Norwalk agent) (Geldreich 1972, Bitton 1999, Mahin and Pancorbo 1999). In addition to vomiting and diarrhea, enteric viruses can cause paralysis, jaundice, meningitis, respiratory illness, liver damage, nephritis, myocarditis, pericarditis, conjunctivitis, fever, and skin rash.

Numbers and types of viruses found in sewage and feces

Approximately 140 different viruses are excreted in human feces and urine (Rao and Melnick 1986, Bitton 1999). Feces from infected individuals may contain as many as 10^6 to 10^{10} picornaviruses per gram (Yates et al. 1985, Rao and Melnick 1986), 10^9 to 10^{10} rotaviruses per gram (Bitton 1980, Hurst and Gerba 1980, Rao and Melnick 1986), and 10^{11} adenoviruses per gram (Bitton 1999). Hepatitis A and polioviruses are excreted in feces over a relatively long period of time, with polioviruses excreted for up to 50 days in man (Fox 1976).

* Formerly referred to as enteroviruses.

Table 2. Major groups of animal DNA[a] viruses. (From Bitton 1980[b].)

Capsid symmetry			**Cubic**		**Complex**
Virion: Naked or enveloped		Naked		Enveloped	Complex coat
Site of capsid assembly		Nucleus		Nucleus	Cytoplasm
Reaction to ether (or other liquid solvent)		Resistant		Sensitive	Resistant
Diameter of virion (*nm*)	18–26	45–55	70–90	100	230–300
Group	Parvoviruses	Papovaviruses	Adenoviruses	Herpesviruses	Poxviruses

[a] All DNA viruses of vertebrates have double-stranded DNA, except members of the parvoviruses, which have single-stranded DNA.

[b] Adapted from Melnick (1976).

Table 3. Major groups of animal RNA[a] viruses. (From Bitton 1980[b].)

	Cubic					**Helical (or unknown)**		
Virion: naked or enveloped	Naked			Enveloped		Enveloped		
Site of capsid assembly	Cytoplasm			Cytoplasm		Cytoplasm		
Reaction to ether (or other liquid solvent)	Resistant			Sensitive		Sensitive		
Diameter of virion (*nm*)	20–30	75	64–66	60	40	80–120	150–300	60–180
Group	Picorna-virus	Reovirus	Rotavirus	Rubella	Arbovirus	Myxovirus	Para-myxovirus	Rhabdo-virus

[a] All RNA viruses of vertebrates have single-stranded RNA, except members of the reovirus group, which are double-stranded.

[b] Adapted from Melnick (1976).

Raw wastewater often carries picornavirus loads of 10^2 to 10^4 plaque-forming units (pfu) per L (Shuval 1976, Irving and Smith 1981). Table 4 gives concentration of several types of viruses found in raw sewage in Australia. Viruses are also shed in large quantities in the feces of healthy carriers, and their number in raw municipal sewage may range from 10^3 to 10^6 pfu/L (Bitton 1980).

The minimal infective doses of some viruses are given in Table 5 (Westwood and Sattar 1976). In many cases there is strong evidence that one virus particle is capable of establishing infection in cell culture or in mammalian hosts (Westwood and Sattar 1976, Bitton 1980).

Table 4. Concentrations of enteric viruses in the raw sewage wastewater of Melbourne, Australia. (From Irving and Smith 1981.)

Type of virus	Mean concentration (*IU/Liter*)*	Range (*IU/Liter*)
Picornaviruses	1,400	150–6,350
Adenoviruses	1,950	0–6,850
Reoviruses	2,150	0–6,150

* IU = Infectious Units

Table 5. Minimal infective doses of some viruses in man. (From Westwood and Sattar 1976.)

Virus	Dose	Unit
Poliovirus 1 (SM)	2.0	pfu
Poliovirus 3 (Fox 13)	10.0	TCD_{50}
Coxsackievirus A21	18.0	TCD_{50}
Coxsackievirus B4	1.3	mouse LD_{50}
Rhinovirus	1.0	EID_{50}
Yellow fever	5.0	mouse LD_{50}

Effects of freezing and low temperature

Lab and field studies (Shuval 1976; O'Brien and Newman 1977; Hurst et al. 1980, 1989; Yates et al. 1985; Gould 1999) have shown that many viruses can persist for months in water and soil during cold weather, and that viral survival is actually greater at low temperatures (~4 to 5° vs. ~22°C) (Ward and Ashley 1976; O'Brien and Newman 1977; Tierney et al. 1977; Bitton 1980; Hurst et al. 1980, 1989). As an example, the time necessary for a 99.9% decline in virus titers in seawater is approximately 40 to 90 days at 3°to 5°C vs. 2.5 to 9 days at 22° to 25°C (Bitton 1980).

Even at subfreezing temperatures, survival is greater than at warmer ambient temperatures. Hurst et al. (1989) found that while there was pronounced inactivation of three human enteroviruses (Coxsackievirus, echovirus, and poliovirus) in surface freshwater at 22°C (the average inactivation was 6.5 to 7.0 log units

over eight weeks), losses were reduced at 1°C, and greatly reduced at –20°C (i.e., average inactivation was four to five log units over 12 weeks at 1°C vs. only 0.4 to 0.8 log units over 12 weeks at –20°C). The number of days required for 90% inactivation (T_{90}) at –20°C ranged from 133 days for poliovirus to 255 days for Coxsackievirus. In activated sludge, the time required for a 90% decrease in titer of enteric viruses was 26 days at 23°C, 180 days at 2°C, and 163 days at –70°C (Hurst and Goyke 1986).

Gould (1999) noted that many cryopreserved viruses have survived for decades at –70°C without the need for special preservation and attributed this stability to the virus's simple structure, small size, and the absence of free water. However, it is not clear if all viruses show the same absence of free water. According to Greiff and Rightsel (1966) and Rightsel and Greiff (1967), some viruses contain some tightly held water. For example, in the southern bean mosaic virus and T2 bacteriophage, water is present in the form of rays or spokes that extend from the inner core of the nucleic acid to the surface of the virus (Greiff and Rightsel 1966, Rightsel and Greiff 1967). However, there is also evidence that the nucleic-acid cores of the T2 phage and influenza virus are immersed in an aqueous phase (Greiff and Rightsel 1966, Rightsel and Greiff 1967). If not all the water in nucleocapsid cores is tightly bound, then presumably this could affect the susceptibility of viruses to freezing.

Greiff (1969) noted that for viruses to be maintained for significant lengths of time, they must be cooled to and stored at temperatures at which biochemical processes responsible for degradation are arrested or greatly retarded or they must be dried sufficiently to bring about the same effect. While some viruses can be frozen and stored at low temperatures with either little or no losses in titers, others cannot (Rightsel and Greiff 1967).

Greiff (1969, 1973) and Rightsel and Greiff (1967) examined the susceptibility of animal viruses to freezing as a function of structural and physico-chemical properties of the viruses. These properties included the type of nucleic acid, and whether the virus has peripheral structural lipids, is enclosed within an envelope or is naked, and is sensitive to low pH (3.0 for 30 minutes) or heat (50°C for 30 minutes). By using these criteria, they were able to place the animal viruses into nine groups (Table 6). Members of each of these groups are listed in Tables 7 and 8.

Table 6. Groupings of viruses based on physico-chemical characteristics. (From Greiff 1969.)

	Characteristics				
Group	Nucleic acid	Solvent	Limiting membrane	pH	Heat sensitivity
I	RNA	Sensitive	Envelope	Labile	Labile
IA	RNA	Sensitive	Envelope	Stable	Labile
II	RNA	Resistant	Naked	Labile	Labile
III	RNA	Resistant	Naked	Stable	Labile
IV	RNA	Resistant	Naked	Stable	Stable
V	DNA	Sensitive	Envelope	Labile	Labile
VI	DNA	Sensitive	Envelope	Labile	Stable
VII	DNA	Resistant	Naked	Stable	Labile
VIII	DNA	Resistant	Naked	Stable	Stable

The viruses were subjected to slow freezing in a basic salt medium, with and without cryoprotectants, by immersion in baths at –40°C or –76°C. For cyclic freezing and thawing, suspensions that were frozen at –76°C were immediately thawed under running tap water at +10°C. To test for the effects of thermal assault, eight cycles of freezing and thawing were used. To test the effects of frozen storage and crystal growth during storage, samples were frozen at either –40°C or –76°C and then stored at either –20°C or –65°C for 30 days. Decreases in infectivity were used as a measure of cryotolerance. Decreases in infectivity titers were evaluated as slight (>0.3 to 0.5 log), moderate (>0.5 to 1 log), marked (>1 log), or extreme (activity was lost or too low to be measured accurately).

Greiff (1969, 1992) found that viruses belonging to the groups I and VIII were cryolabile, even if cryoprotective compounds were added to the basic suspending media (Tables 9, 10). In contrast, viruses belonging to the groups II, III, V, and VI were not changed appreciably by freezing or storage at low temperatures (Tables 9, 10). For RNA viruses, those that did not have a lipid coat or envelope were more resistant to freezing and storage at low temperatures than those that did (Table 9). Also for RNA viruses, pH stability did not correlate with cryotolerance. In contrast, DNA viruses that possessed a lipid coat were more cryotolerant than those that did not posses an envelope. Also, DNA viruses that were pH labile were cryotolerant, while those that were pH stabile were cryointolerant.

There does not appear to be much information on the effect of freezing rate on the survival of viruses other than a study by Hurrell (1967), who reported that there did not appear to be any advantage to either slow or rapid freezing for

Myxoviruses. According to Greiff (1969, 1992), these viruses are not freeze-tolerant.

From this work, we can conclude that several viruses of concern, including the picornaviruses, can withstand freezing. The cryosensitivity of many others still needs to be determined, as does the effect of freeze–thaw cycling.

Table 7. More common RNA viruses belonging to the groups classified according to physico-chemical characteristics. (From Greiff 1969.)

Group I	Measles
Arboviruses	Canine distemper
Group A	Rabies virus
Semliki Forest	Fowl lymphomatosis virus
Sindbis	Rous sarcoma
Western equine encephalitis	Others (unique)
Group B	Rubella
Dengue	Group IA
St. Louis encephalitis	Vesicular Stomatitis virus
West Nile	Group II
Yellow fever	**Picornaviruses**
Russian spring–summer encephalitis	**Foot and mouth disease virus**
Ungrouped	**ECHO (selected)***
Blue tongue	**Salisbury agents**
Colorado tick fever	**Coryzaviruses**
Rift Valley fever	**Group III**
Turlock	**Picornaviruses***
Myxoviruses	**Poliovirus***
Influenza virus, type A	**Coxsackie***
Influenza virus, type B	**ECHO (selected)***
Swine influenza	**Vesicular exanthema of swine**
Paramyxoviruses	Group IV
Mumps	Picornaviruses
Newcastle disease virus	Porcine enteroviruses
Parainfluenza	Columbia SK
Hemadsorption 1	Mengo
Other myxoviruses	Reoviruses*
Respiratory syncytial virus	Serotypes

* Viruses found in wastewater

Groups in bold type were not affected by freezing.

Table 8. More common DNA viruses belonging to the groups classified according to physico-chemical characteristics. (From Greiff 1969.)

Group V
Herpesviruses
Subgroup A
Herpes simplex
B virus (monkeys)
Equine herpes virus
Infectious laryngotracheitis
Subgroup B
Varicella-zoster
Cytomegalovirus
Inclusion virus (man or guinea pig)
Group VI
Poxviruses
Variola
Vaccinia
Fowl pox
Myxoma (rabbit)
Molluscum contagiosum (man)
Group VII
Adenoviruses*
Human serotypes
Infectious canine hepatitis
Simian serotypes
Avian
Murine
Group VIII
Papovaviruses
Shope papilloma virus
Polyma (mouse)
Vacuolating agent (SV-40)
RKV virus (rabbit kidney vacuolating)
* Viruses found in wastewater
Groups with bold type are resistant to freezing.

Table 9. Effects of freezing, cyclic freezing and thawing, and storage at low temperatures on the titers of selected RNA viruses classified according to physicochemical characteristics.* (From Greiff 1969.)

Experimental treatment	Group I Respiratory syncytial (RS), influenza, measles, rubella	Group IA Vesicular stomatitis virus[3]	Group II Foot and mouth disease virus	Group III ECHO 9, poliovirus
Frozen	Slight decreases	BSM-Moderate to marked decreases PDA-No change	No change to slight decreases	No change
Cyclic freezing and thawing	Slight to marked decreases	BSM-Moderate decrease PDA-No change	BSM-No change PDA-Moderate decrease	No change
Nonfrozen; stored at +4°C for 30 days	RS, influenza, measles: Extreme decreases BSM>DMSO>PDA Rubella: Marked decrease BSM>DMSO>PDA	Moderate decreases	Slight decreases	No change
Frozen –40°C; stored at –20°C for 30 days	RS, influenza, measles; Moderate to extreme decreases BSM>DMSO>PDA Rubella: Marked decrease	Marked decreases BSM>PDA	No change	No change
Frozen –40°C; stored at –65°C for 30 days	RS: Extreme decrease Influenza: No change Measles: Slight decrease BSM>DMSO PDA-No change Rubella: BMS, PDA-Marked decrease DMSO: Moderate decrease	BSM-Moderate decrease DPA-Marked decrease	No change	No change
Frozen –76°C; stored at –20°C for 30 days	Marked to extreme decreases	Marked decrease	No change	No change
Frozen –76°C; stored at –65°C for 30 days	RS: Extreme decrease Influenza: No change Measles, rubella: Moderate decrease	Marked decrease	No change	No change

* Based on data of O.N. Fellowes (1968) and Rightsel and Greiff (1967).

Table 10. Effects of freezing, cyclic freezing and thawing, and storage at low temperatures on the titers of selected DNA viruses classified according to physico-chemical characteristics. (From Greiff 1969.)

Experimental treatment	Group V Herpes simplex	Group VI Vaccinia	Group VIII Vacuolating agent (SV-40)
Frozen	No change	No change	No change
Cyclic freezing and thawing	No change	No change	No change
Nonfrozen; stored at +4°C for 30 days	Slight decrease	Slight decrease	Slight to moderate decrease DMSO<PDA<BSM
Frozen –40°C; stored at –20°C for 30 days	Slight decrease	No change	Slight to moderate decrease BSM<DMSO<PDA
Frozen –40°C; stored at –65°C for 30 days	No change	No change	No change
Frozen –76°C; stored at –20°C for 30 days	No change	No change	Moderate decrease
Frozen –76°C; stored at –65°C for 30 days	No change	No change	No change

Viral sorption on solids

Viruses have an affinity for suspended solids in the aquatic environment, such as silts, clay minerals, cell debris, or particulate organic matter. Wellings et al. (1976) found that 23% to 99.99% of the viruses in raw sewage were associated with the solids. The kinetics of virus adsorption depends upon a number of variables including the nature of the suspended solids (e.g., concentration, whether organic or inorganic, size, particle charge, availability of attraction sites), the metal cation concentration and valence, the nature of the virus (e.g., the isoelectric point, whether enveloped), pH, and the presence of organic or inorganic interfering substances (Schaub and Sorber 1976, Bitton 1980, Hejkal et al. 1981). Monovalent, divalent, and trivalent cations at the same molarity generally have sequentially greater flocculating capability and thus virus adsorbing ability (Schaub and Sorber 1976).

Solids-associated viruses reportedly survive longer in natural water and may settle and accumulate in the sediments, where viral survival is also longer than in the overlying water (Gerba et al. 1977, Smith et al. 1978, Bitton 1980, Hurst et

al. 1989). Enteric viruses also generally survive longer in the presence of sewage effluents (Dahling and Safferman 1979, Hurst and Gerba 1980). However, Hurst and Gerba (1980) noted that this will vary depending upon the relative affinity of the virus for the particulate matter in the effluent. The increased survival of viruses associated with particulate matter may be the result of the stabilizing effect of organic materials and the protective effect of inorganic and organic colloids present in wastewater (Bitton 1978). Clay minerals generally protect viruses from biological (enzyme action) and physico-chemical (temperature, pH, ultraviolet and visible light) inactivation (Bitton 1980). Animal viruses sorbed to clay particles have also been shown to retain their infectivity (Schaub and Sagik 1975).

In addition, enteric viruses released from the infected host are in a highly aggregated state and probably remain so upon entering wastewaters and natural waters (Bitton 1980). Aggregation also results in increased survival of viruses in natural waters (Bitton 1980).

However, Cubbage et al. (1979) cautioned that either viral aggregation or sorption of viruses on particulate matter in aqueous samples could result in underreporting viral concentrations.

Survival in cold environments–Field studies

We were unable to find any documentation of the recovery of enteric viruses from the Arctic or Antarctic.

Conclusions

Although there is not any documentation of the long-term survival of viruses in frozen environments, many viruses, including many of those of concern (the picornaviruses), can survive being frozen for months in the environment or for decades in a laboratory freezer at –70°C (Gould 1999). Studies on classifying the cryosensitivity of viruses based on physico-chemical characteristics have proven useful and should be pursued further to determine what other species of enteric viruses can withstand freezing. More research is needed to determine the effects of repeated freeze–thaw cycling.

5 PROTOZOA

General information

Over 15,000 species of protozoa have been described. Generally, they are unicellular animals and are microscopic in size, although a few can be seen with the naked eye. However, in spite of their small size, their structure is complex. They contain one or more nuclei and a number of other structures (organelles). Organelles can include a cytostome (cell mouth for ingestion of food), a cytophage (cell anus for elimination of waste matter), sucking tentacles (for absorbing food), contractile vacuoles (which serve as hydrostatic regulators), food vacuoles (for digesting food), and pigment spots (that give light sensitivity and a sense of taste). Figure 8 shows the typical structure of a paramecium protist.

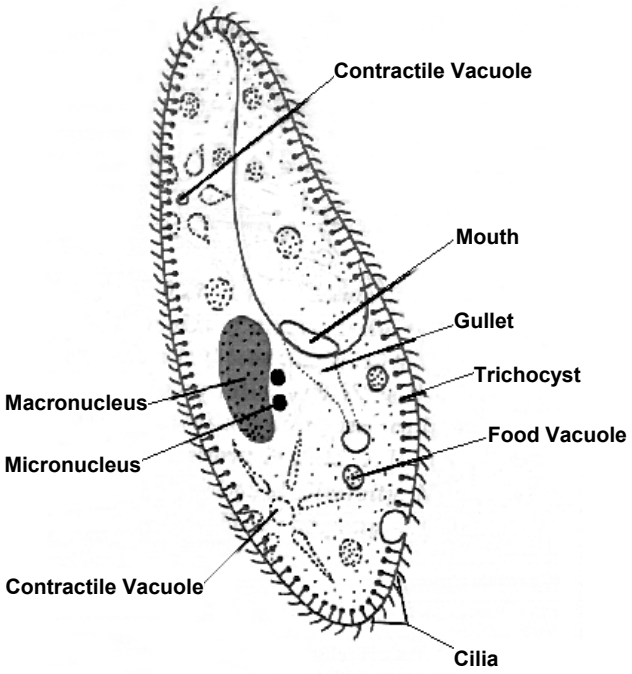

Figure 8. Diagram of a paramecium cell. (After Brock 1970.)

A large number of protozoa are parasitic on animals, including man. Wastewater-borne protozoa pathogenic to man include *Giardia lamblia*, *Cryptosporidium* sp., *Balantidium coli*, *Cyclospora cayetanensis, Entamoeba histolytica,* and *Toxoplasma* spp. Gastro-intestinal symptoms include diarrhea, constipation, abdominal pain and cramps, bloody stools, nausea, dehydration, and weight loss.

Most protozoa are motile and their form of motility is used for classification (Table 11). Forms of locomotion include amoeboid movement (the Sarcodina), the use of flagella (the Mastigophora), and the use of cilia (the Ciliophora). Members of a fourth class (the Sporozoa) are parasitic on higher animals and are usually nonmotile. Members of a fifth class (the Suctoria) are sessile organisms with suctorial tentacles (Fox et al. 1981).

Although most protozoa lack a true cell wall, they do have some type of surface layer, known as a pellicle or cortex, which provides an element of rigidity and gives them definitive shape. In ciliates, the cortex is thick and contains a variety of structures. Many protozoa, especially the Sarcodina, produce shells of cellulose, chitin, cemented sand grains, silica, lime, or other substances. Some flagellates and ciliates have skeletal layers, called loricas or tests, which provide protection and support.

Reproduction can be asexual or sexual. Asexual reproduction may involve simple fission, budding, or multiple-fission, and may result in the formation of cysts. At some time in their life cycle, many protozoa are able to form cysts, which are relatively impervious protective capsules around their bodies. This enables them to survive unfavorable environmental conditions such as desiccation, unfavorable temperatures, injurious chemicals, or lack of oxygen, and aids in dispersal of the species. For the species of protozoa typically found in sewage, encystment occurs in the intestinal tract of the host. In Sporozoa, cyst formation is associated with sexual reproduction and the fertilized gametes are enclosed in oocysts (in which multiplication occurs and can result in the formation of a few to thousands of sporozoites).

Numbers and types found in sewage and feces

Infected individuals can shed up to 5×10^6 *Giardia lamblia* cysts/gram and 10^9 *Cryptosporidium* cysts/gram in feces (Bitton 1999). Concentrations in raw wastewater can range from hundreds to 10^5 *Giardia lamblia* cysts/L, from 850 to 13,700 *Cryptosporidium* cysts/L, and up to 5,000 *Entamoeba histolytica* cysts/L (Bitton 1999).

A survey of 66 North American Water treatment facilities showed 78% of source water and 27% of treated water were contaminated with oocysts (Graczyk et al. 1997). Wallis et al. (1996) surveyed water samples from 72 municipalities in Canada for the presence of protozoan cysts. They found *Giardia* cysts in 73% of the raw sewage samples, 21% of raw water samples, and 18.2% of treated water samples. However, cyst viability was low. Only 3% of the water samples and 17% of the sewage samples were infectious. Incidence of *Cryptosporidium*

oocysts was much lower: 6.1% in the sewage samples, 4.5% in the raw water samples, and 3.5% in the treated water samples.

Table 11. Classes of Protozoa and species found in Chicago's sewage. (From Fox et al. 1981.)

Class	Common Name	Description	Sewage species
Sarcodina	Amoebae	Move by means of pseudopodia	*Entamoeba coli* *Chochlipodium* sp. *Amoeba* sp. *Acanthamoeba* sp. *Naegleria* sp. *Valkampfia* sp. *Arcella* sp. *Euglypha* sp. *Centropyxis* sp. *Difflugia* sp. *Corythion* sp.
Mastigophora	Flagellates	Have one or more flagella for locomotion	*Giardia* sp. *Giardia lamblia* *Bodo* sp. *Trepomonas* sp. *Monas* sp. *Tetramitis* sp. *Rhynchomonas* sp. *Peranema* sp.
Sporozoa	Sporozoans	Spore forming, no organelles for locomotion, intracellular parasites	*Eimeria* sp. *Isospora* sp.
Ciliata	Ciliates	Move by means of cilia	*Trachelophyllum* sp. *Amphileptus* *Busaridium* sp. *Tetrahymena* sp. *Colpodium* sp. *Glaucoma* sp. *Leukophrys* sp. *Chilodonella* sp. *Litonotus* sp. *Euploytes* sp. *Aspidisca* sp. *Stentor* sp. *Vorticella* sp. *Epistylis* sp. *Zoothamnium* sp. *Actinophrys* sp.
Suctoria		Sessile organisms with suctorial tentacles	*Tokophyra* sp. *Acineta* sp. *Podophyra* sp. *Discophyra* sp.

While a single human stool can contain 10^6 to 10^9 cysts, as few as 10 *Giardia lamblia* cysts (Bitton 1999) or 10 to 30 *Cryptosporidium* oocysts (Bitton 1999, Carodona 2000) may be all that are necessary to cause infection.

Effects of chilling, cold shock, and cold adaptation

There is substantial information on the effects of rapid chilling (cold shock) on the free-living forms but considerably less information on the effect of cold shock on the cysts.

Many protozoa are eurythermic (i.e., their vital activity continues within a wide range of fluctuation of the ambient temperature) and some species are cryophilic (i.e., adapted to life at low temperatures) (Lozina-Lozinskii 1974). The hardiness of protozoa to very low temperatures depends upon whether they are encysted or in an active state and whether they belong to a particular systematic or biological group (Lozina-Lozinskii 1974).

According to McLellan et al. (1984), strains of amoeba have been used to study the mechanism of cellular injury by rapid cooling (cold shock). Cell viability was found to depend on the time and temperature of cold exposure, on the rate of cooling, and on the morphology of the cells prior to chilling.

Rapid cooling to 0°C provokes injury in Infusoria and shock reaction in amoeba while with gradual cooling, adaptation is observed (Lozina-Lozinskii 1974). There are a number of changes that occur during gradual cooling. The viscosity of the protoplasm increases with decreasing temperature, especially with amoeba (Lozina-Lozinskii 1974). Cells of Infusoria that have been kept at 0°C deplete stored fat and accumulate glycogen (Lozina-Lozinskii 1974). The glycolytic type of metabolism begins to predominate and influences the size of the organism (Lozina-Lozinskii 1974). Also at low temperatures, an increase in the amount of RNA and DNA has been observed with *Paramecium caudatum* (Lozina-Lozinskii 1974). Lozina-Lozinskii (1974) concluded that the biochemical adaptive reactions of protozoa correspond to similar reactions in invertebrate animals and in part to heterothermic vertebrates.

Effect of freezing on free-living forms

Free-living species of protozoa (e.g., flagellates, and amoebae) can be cryopreserved but require cryoprotectants (e.g., 5% DMSO) to do so (Diamond 1964, Lozina-Lozinskii 1974). However, some parasitic protozoa, such as malaria parasites that inhabit the blood, can be cryopreserved without using a cryoprotectant (Diamond 1961, Lozina-Lozinskii 1974).

Survival is highest with slow or stepwise cooling (Diamond 1961, Lozina-Lozinskii 1974). Lozina-Lozinskii (1974) noted that this agrees with Mazur (1964) and that it must be assumed that with slow cooling the cells survive as a result of dehydration. Dehydration may proceed extremely rapidly as a consequence of the small dimensions of these organisms and the high permeability of their outer membrane to water (Lozina-Lozinskii 1974). Some protozoa also endure ultrarapid cooling, on the condition that they are warmed equally rapidly (Lozina-Lozinskii 1974). Lozina-Lozinskii (1974) believes that in these cases, vitrification takes place and there is no recrystallization of the ice. Extremely slow rates of cooling, rates so slow that several hours are required to pass from room temperature to –79°C, also have been used successfully (Diamond 1961).

Frozen protozoa have been stored successfully at temperatures as high as –19°C and as low as –196°C (Diamond 1961). As expected, survival is generally longer at lower storage temperatures (Diamond 1961). Water usually freezes within the cells of protozoa that have been frozen slowly but not to a low temperature and will perish after even rapid warming (Lozina-Lozinskii 1974). The resistance of protozoa to ultralow (<–79°C) and low temperatures depends upon many factors, including the amount of time spent at a given temperature. For some, the time factor scarcely plays a role, indicating complete stabilization of the cell, while for others it is significant (Lozina-Lozinskii 1974).

After warming frozen Infusoria, ruptures of the pellicule and macronucleus are frequently observed (Lozina-Lozinskii 1974).

Effects of freezing on cysts

Marquardt et al. (1966) found that cysts of *Colpoda steinii* (a ciliated protozoan) and *Vahlkampfia* sp. (an amoeba) survived freezing at –28°C and at –95°C, but free-living forms did not.

As a rule, cysts can endure very low temperatures, especially if they are partially desiccated (Lozina-Lozinskii 1974). For example, dehydrated cysts of *Colpoda cucullus* survived cooling to –196°C (Lozina-Lozinskii 1974). However, in an aqueous environment the cysts are less resistant to very low temperatures because under certain conditions, the water that is contained within them may crystallize out (Lozina-Lozinskii 1974). This is partially counteracted by the partial loss of water and increase in viscosity of the protoplasm (Lozina-Lozinskii 1974). Also, the wall of the cyst acts as a barrier, preventing penetration of ice crystals from outside the cyst (Lozina-Lozinskii 1974).

Lozina-Lozinskii (1974) studied the freezing of dormant cysts of *Colpoda maupasi.* With slow chilling (1.5°C per minute), the cysts supercooled to –10°C

or –12°C, but after freezing of the medium, intracellular formation of ice occurred. On freezing, the cysts shriveled and diminished in size, indicating extracellular freezing. The form of the cysts did not change on further slow cooling to –78° C and –196° C. With very rapid freezing (–70°C in 1 to 2 seconds), the cysts did not shrivel and crystallization and recrystallization could be observed within the cells. With ultrarapid cooling to –196°C, water froze inside the cells. The survival rate of the cysts that were cooled rapidly varied from 22% after warming at 30°C to 50% at 60°C. However, all the cysts were dead after thawing at room temperature. They attributed the higher survival rate at the highest warming temperature to the fact that recrystallization was averted. At the start of freezing the cysts are transparent and there is no deformation. From the moment of warming, the protoplasm darkens, indicating the growth of crystals.

Robertson et al. (1992) exposed aqueous suspensions of *C. parvum* oocysts to snap freezing (immersion in liquid nitrogen) and slow freezing (placed in a freezer at –22°C). Specimens were defrosted by thawing at 4°C for two to three hours. Ultrarapid (snap) freezing resulted in 100% death while slow freezing was less effective in killing oocysts. In this case, the warming rate was probably not rapid enough to allow for survival of the snap-frozen oocysts. After 32 days, only 2% of the slowly cooled oocysts were still viable.

While these results agree with Mazur's (1964) observations/predictions regarding slow and rapid freezing, Fayer and Nerad (1996) found that aqueous suspensions of *Cryptosporidium parvum* oocysts retained viability and infectivity after freezing and actually survived longer at higher freezing temperatures. Oocysts were frozen at –10°C, –15°C, –20°C, and –70°C for up to 168 hours and then thawed at room temperature (21°C). They found that oocysts that had been frozen at the warmest temperature were infectious, while those frozen at –70°C were not. Oocysts frozen at –10°C for 168 hours were still infectious while those frozen at –20°C were infective only if storage time was eight hours or less. It may be that those "frozen" at –10°C were only supercooled and not frozen, while those subjected to lower temperatures actually did freeze. It is also possible that the rate of warming was too rapid for these organisms.

Sanin et al. (1994) found that oocysts of *C. parvum* oocysts in sludge samples were completely destroyed (>8-log reduction) by freezing at –25°C for seven days.

As expected, multiple freeze–thaw cycles have a much more negative impact than a single cycle of freezing and thawing. Erlandsen et al. (1990) studied changes in cysts of *Giardia muris* and *Giardia lamblia* that had been subjected to one to three cycles of freezing at –16°C and thawing at 20°C. Examination

using immunofluorescence showed that multiple freeze–thaw cycles frequently distorted the cysts, changing them from oval to elongated shapes with blunt ends. Examination of *G. muris* cysts by high resolution SEM demonstrated that the distorted elongated cyst was partially collapsed and that the filamentous cyst wall appeared disassociated rather than tightly packed. They observed that additional freezing and thawing resulted in additional losses and that losses were less at higher cyst concentrations (Table 12).

Table 12. Percentage of cysts of *Giardia muris* recovered after one and three cycles of freezing and thawing. (From Erlandsen et al. 1990.)[a]

	Number of freeze–thaw cycles	
Initial cyst concentration per mL	**1**	**3**
1.4×10^6	94	78
4.6×10^5	102	73
8.9×10^4	41	32
2.4×10^4	58	17

[a] From –16°C to –20°C.

Survival in cold environments–Field studies

There is relatively little information on the long-term survival of protozoa in the environment (Rose 1997). DeRegnier et al. (1989) studied the short-term viability of *Giardia muris* cysts at low temperatures. In the fall, cysts suspended in lake water remained viable for 28 to 56 days. In contrast, cysts remained viable for longer in the winter, 56 to 84 days. Comparison with water quality parameters (temperature, pH, dissolved oxygen, turbidity, color, hardness, ammonia, nitrate, phosphorous) revealed that only decreased water temperature (<10°C) correlated with prolonged survival of these organisms.

Robertson et al. (1992) observed that *C. parvum* oocysts recovered from feces at 4°C had developed an enhanced impermeability to small molecules. They felt that this might increase their robustness when exposed to environmental pressures, such as freezing. They also noted that oocysts demonstrated longevity in all types of water, including seawater.

Marquardt et al. (1966) recovered *Colpoda steinii* (a ciliated protozoan) and *Vahlkampfia* sp. (an amoeba) from an ice tunnel in Greenland. Given that the ice in the tunnel was at least several hundred years old, the survival of these organisms may represent the longest known survival of protozoan species. Marquardt et al. (1966) noted that cysts of *Colpoda* have been found to survive for as long as 38 years in dry soil.

Conclusions

Although there is ample evidence that oocysts of some species, especially *Colpoda* spp., can withstand freezing, it is not clear which classes of protozoa survive best. However, there is some evidence (Sanin et al.1994, Fayer and Nerad 1996) that *Cryptosporidium* oocysts do not withstand slow freezing and that cyclic freeze–thawing at temperatures between –16°C and +20°C injures *Giardia* species. It is also not clear whether oocysts will be infectious after being frozen for decades or longer. Very few studies have mimicked the slow freezing and thawing that would occur in nature.

6 HELMINTHS OR PARASITIC WORMS

General information

Helminths are the largest life form considered in this paper and vary considerably in their life cycle. Some have only one host and reproduce only by conventional sexual means. Others have elaborate life cycles that can include a number of asexual stages that can involve a number of intermediate hosts. The parasitic helminths belong to four phyla of the animal kingdom: the Platyhelminthes or flatworms, the Acanthocephala or spiny-headed worms, the Nemathelminthes (or Aschelminthes) or roundworms, and the Annelida (or Annulata) or segmented worms. Members from the first three phyla typically deposit eggs, and in some instances also deposit larvae or adults, in feces or urine and thus are of interest in this review. A more detailed description of the organisms in these phylum can be found in Appendix A. Species of interest include the tapeworms *Taenia solium* and *Taenia saginata* (beef tapeworm); the nematodes *Enterobius vermicularis* (pinworm)*, Trichuris trichiura* (whipworm), and *Ascaris lumbricoides* (roundworm); and the hookworms *Ancylostoma duodenale* and *Necator americanus*.

Many helminth infections can be attributed to eating foods that haven't been cooked properly or to conditions of poor sanitation. Many helminths that typically infect other species such as dogs, cats, fish, cattle, and pigs can also incidentally infect humans. Therefore, human feces can contain species not normally attributed to man. Infection may lead to clinical symptoms and a disease state, but often hosts are unaware that they carry the parasite. Clinical symptoms can include fever, chest pain, diarrhea, vomiting, nutritional deficiencies, neurological problems, weight loss, and muscle aches.

In many instances, whole groups of worms have common egg characteristics. For example, the ova of tapeworms (of warm-blooded animals) contain a fully developed six-hooked embryo. Eggs vary in shell thickness. Members of the tapeworm family have thick inner shells, ascarids have thick shells with surface markings, and hookworms and their allies (Stronglylata) have thin shells.

Ova with thicker shells are typically more resistant to adverse environmental conditions, including sewage treatment. Eggs of *T. trichiura* are known to survive for months in contaminated soil, and eggs of *A. lumbricoides* can survive for several years despite drying or adverse chemical conditions (Lewert 1968). Thick-shelled ova that are typically resistant to sewage treatment practices include the ascarids (*A. lumbricoides*, *A. lumbricoides* var. *suum*, *Toxocara*

canis), the whipworms (*Trichuris* sp.), the tapeworms (*Taenia* sp.), and Hydatid (*Echinococcus* sp.) (Fox et al. 1981). Researchers have found that among the helminths, the ova of the ascarid roundworms are the most resistant to adverse environmental changes (Fox et al. 1981).

Numbers found in sewage

The female *A. lumbricoides* can discharge 200,000 eggs/day in the feces, and the adult tapeworm *T. saginata* can discharge 10^6 eggs/day in the feces (Geldreich 1972, Bitton 1999). Raw sewage of Darmstadt, Germany, contained 540 eggs of *Ascaris*/100 mL (Geldreich 1972). In contrast only a few (1 to 10) eggs of *A. lumbricoides* can cause infection (Bitton 1999, Carodona 2000).

Effects of cold and freezing on ova, larvae, and adults

Generally, there is less information available on the effects of low temperature and freezing on these organisms. However, there is some evidence that the survival of larvae is enhanced at low temperatures. Shostak and Samuel (1984) found that the 50% survival time of first-stage larvae of *Parelaphostrongylus odocoilei* in water was 232 days at 5°C vs. 23 days at 26°C.

Sanin et al. (1994) found that the thick-shelled *Ascaris* ova were virtually unaffected when frozen (–25°C) in sludge from wastewater treatment plants. We were unable to find any additional studies that examined the effects of freezing on helminth ova. There are, however, several papers on the ability of the larvae and adults to survive freezing.

Shostak and Samuel (1984) found that first-stage larvae of *Parelaphostrongylus* spp. (*P. odocoilei* and *P. tenuis*) (which are shed in the host's feces) had minimal mortality after being frozen in water at –25°C for 280 days.

According to Snyder (1985), there have also been numerous reports of first-stage larvae surviving frozen storage in animal scats. These include *Protostrongylus* spp. for up to 20 months at temperatures as low as –40°C (Forrester and Senger 1963), *Pneumostrongylus tennuis* for 306 days at –15° to –20°C (Lankester and Anderson 1968), and *Skjabingylus nasicola* for 77 days at –20°C (Hansson 1974). There also have been reports of microfilariae and adult nematodes (*Pelecitus fulicaeatrae*) surviving in carcasses of their host birds for three to five months at –21°C to –24°C (Bartlett 1992). However, many of the first-stage larvae that survived freezing lost their infectivity to the intermediate host (Snyder 1985).

According to Nolan et al. (1988), studies have shown that larvae of some species can be cryopreserved using liquid nitrogen, and cryoprotectants are not

required for some of the nematode larvae. However, they also noted that while the larvae of Strongyloides spp. were not infective after freezing, many parasitic nematodes (*Haemonchus contortus*, *Ancylostoma caninum*, and those of ruminants) retained their infectivity.

Shostak and Samuel (1984) noted that while long-term frozen storage of first-stage larvae of *P. odocoilei* and *P. tenuis* (in water) had minimal impact on mortality, repeated freeze–thaw cycling (+14°C to –25°C, two times a day) decreased survival, especially when there were ten or more cycles (Table 13). They believe that the distribution of these parasites depends in part on the ability of their free-living stages to survive variable environmental conditions such as freezing.

Table 13. Survival of first-stage larvae of *P. odocoilei* and *P. tenuis* following repeated freezing. (From Shostak and Samuel 1984.)

Number of freeze–thaw cycles	*P. odocoilei* Mean % survival	*P. tenuis* Mean % survival
0	98 ± 2.6	99.7 ± 0.6
1	98.5 ± 0.5	95.9 ± 1.4
10	79.5 ± 4.5	66.4 ± 4.7
20	11.8 ± 2.4	4.5 ± 0.7

Cold adaptation

Bartlett (1992) postulated that because the host birds of *Pelecitus fulicaeatrae* were subjected to very cold water and sub-freezing air temperatures, those conditions might have selected for cold-hardy strains of this organism. Bartlett (1992) noted that although reports of cold-hardiness are rare among filarioids, the phenomenon is well known among parasitic nematodes (with free-living stages) of mammals in temperate latitudes. James (1985) also noted that the infective larvae of many nematodes of domestic animals undergo freezing temperatures in winter in temperate latitudes and noted that many of these species can be successfully cryopreserved in tap water with slow cooling. James (1985) reported that arctic isolates of *Trichinella nativa* can tolerate being frozen at –45°C and survive at –15°C for more than two years.

Conclusions

There is evidence that not only the ova of *Ascaris* spp. but also many of the larvae and adults of many types of helminths can withstand freezing. Further studies are needed to determine if all ova are resistant to freezing and whether susceptibility to freezing can be associated with shell thickness and certain groups of helminths.

7 COMPARISONS OF THE SURVIVAL OF VARIOUS TYPES OF MICROORGANISMS

According to Ridgway (1984), in most environments vegetative bacteria are more susceptible to inactivation than viruses, which are more susceptible to inactivation than bacterial spores and protozoa (Ridgway 1984). However, we found relatively few studies that compared the survival of various types of microorganisms in the environment and only one (Sanin et al. 1994) that examined their survival at freezing temperatures. Table 14 gives a compilation of survival times in various environments.

Bitton et al. (1983) compared survival characteristics of three bacteria (*Salmonella typhimurium*, *Streptococcus faecalis*, *Escherichia coli*), a picornavirus (poliovirus type 1), and a bacteriophage (phage f2 for *E. coli*) in groundwater held at 22°C. They found that the poliovirus and *S. faecalis* had the lowest decay rates and that the f2 phage had the highest decay rate.

Keswick et al. (1982) compared the persistence of three animal viruses (Coxsackievirus B, poliovirus type 1, rotavirus SA11 [a simian rotavirus, not a human rotavirus]), a bacteriophage (phage f2), and two bacteria (*Escherichia coli* and a sewage-isolated fecal *Streptococcus*) in groundwater that was held at lower temperatures, 3°C to 15°C. They found that the two human viruses (the Coxsackievirus and poliovirus) had lower decay rates than the bacteria, but the rotavirus and bacteriophage were inactivated at faster rates than the bacteria.

With respect to the resistance of various types of microorganisms in sewage sludge to freezing (–25°C), Sanin et al. (1994) found that *Ascaris* ova were the most resistant to freezing, with almost no loss (Table 15). In contrast, protozoan oocysts (of *Cryptosporidium parvum*) were completely destroyed by freezing (>8-log reduction). A bacteriophage and a poliovirus were less susceptible than fecal coliforms, and *Salmonella* and fecal streptococci were less susceptible than the two viruses tested.

These results agree well with each other and with the previous findings that have shown/predicted the resistance of the picornaviruses (Coxsackie and polio viruses) to freezing, and that gram-positive bacteria (e.g., *S. faecalis*) are more resistant to freezing than gram-negative coliforms, especially *E. coli*.

Table 14. Potential pathogens in raw sewage (from Metcalf & Eddy, Inc. 1991) and survival time (from various references given below).

Microorganism		Disease	Survival time	Medium	Reference
Bacteria	*V. Cholera*	Cholera (extreme heavy diarrhea)	6 weeks 24 hours 16 days 30 days	water containing organic matter sewage soil Anticipated max. life of infective stage at 20–30ºC	Burrows and Renner (1999) Bradley and Feachem (1979)
	Salmonella	Typhoid fever, acute gastroenteritis	29–58 days 9 days 8 days 5 months 60 days to 1 year	soil seawater fresh water ice Anticipated max. life of infective stage at 20–30ºC	Burrows and Renner (1999) Bradley and Feachem (1979)
	Shigella dysenteriae	Shigellosis (dysentary)	2–3 days 40 days	water Anticipated max. life of infective stage at 20–30ºC	Burrows and Renner (1999) Bradley and Feachem (1979)
	Pathogenic *E. Coli*	Gastroenteritis	1 year	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Legionella pneumophila	Legionellosis (acute respiratory illness)		Prefers warm stagnant water	www.dhfs.state.wi.us/healthtips/BCD/Legionellosis.htm
	Leptospira	Leptospirosis (jaundice, fever)		Viable for long periods in freshwater, damp soil, vegetation, and mud	www.astdhpphe.org/
	Yersinia enterolitica	Yersinosis (diarrhea)	6 months	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
Viruses	Adenovirus (31 types)	Respiratory disease			
	Enteroviruses (67 types, e.g., polio, echo, Coxsaxie)	Gastroenteritis, heart anomalies, meningitis	6 months	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Norwalk Agent	Gastroenteritis			
	Hepatitis A	Gastroenteritis			
	Reovirus	Gastroenteritis			
	Rotavirus	Gastroenteritis	8–32 days > 64 days 1 year (?)	surface waters tapwater Anticipated max. life of infective stage at 20–30ºC	Burrows and Renner (1999) Bradley and Feachem (1979)

Table 14 (cont'd).

Microorganism		Disease	Survival time	Medium	Reference
Protozoa	*Balantidium coli*	Balantidiasis (diarrhea, dysentary)	1 month (?)	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Cryptosporidium	Cryptosporidiosis (diarrhea)	18 months	"environment"	Duffy (1999)
	Entamoeba histolytica	Amebiasis (amoebic dysentery)	20 days	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Giardia lamblia	Giardiasis (diarrhea, nausea, indigestion)	3 months	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
Helminths	*Ascaris lumbricoides*	Ascariasis (roundworm infestation)	several years	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Enterobius vericularis	Enterobiasis (pinworm)	7 days	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Fasciola hepatica	Fascioliasis (abdominal pain, fever, vomiting)	10 weeks	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Hymenolepis nana	Hymenolepiasis (weakness, headaches, anorexia)	A few weeks	Anticipated max. life of infective stage at 20–30ºC	Bradley and Feachem (1979)
	Taenia saginata and *T. solium*	Taeniasis (mild abdominal pain)	Up to 25 years	Not stated	www.totaldigestivehealth.com/taeniasis.html
	Trichuris trichiura	Trichuriasis (abdominal pain, diarrhea	1.5 years 15–30 days	Anticipated max. life of infective stage at 20–30ºC soil	Bradley and Feachem (1979) www.totaldigestivehealth.com/trichuriasis.html

Table 15. Reduction of pathogenic and indicator microorganisms in two different sludges by freeze–thaw conditioning. (From Sanin et al. 1994.)

	Overall log reduction	
	Aerobically digested sludge	**Anaerobically digested sludge**
Fecal coliforms	1.90	1.10
Fecal streptococci	0.21	0.20
Salmonella	0.54	0.74
Viral Plaque Forming Units	0.80	0.85
Poliovirus	1.08	1.47
Helminth ova[a]	–0.06	–0.03
Protozoa oocysts[b]	>8.00	>8.00

[a] *Ascaris*
[b] *Cryptosporidium parvum*

8 CONCLUSIONS

Microorganisms that are found in sewage and are of concern to human health include bacteria, viruses, protozoan cysts, and helminth ova. These organisms range in structural complexity from almost lifeless viruses that are simple nucleic acids contained in a protein coat, to the cells of procaryotic bacteria that contain both cell membranes and cell walls and naked DNA in the cell cytoplasm, to more complex eucaryotic organisms that have a cell membrane but no cell wall and that contain DNA in chromosomes in a membrane-bound nucleus. These eucaryotes include the single-celled protozoa and the multicellular helminths with complex life cycles that include the cysts and ova found in sewage. As might be expected based on structural differences, these organisms differ in their susceptibility to freezing and thawing. However, because of all the differences between the various species within each of these life forms, there are no simple hard and fast relationships between freezing and thawing and these life forms. This is probably to be expected as these organisms have evolved over millions of years and those that have been exposed to occasional freezing and thawing have had time to evolve mechanisms to adapt to this adverse environmental threat. Among the viruses, there is research that allows one to predict the susceptibility to freezing and thawing based upon differences in structure, such as the type of nucleic acid, presence of an envelope, and susceptibility to low pH. However, more research is needed to verify those predictions.

Generally, all living cells respond best to an intermediate but relatively slow freezing and thawing rate and extended storage at temperatures below –60°C. Microorganisms in a sewage bulb in the ice of Antarctica would be subjected to freezing and thawing rates that are slower than the optimum rate and a storage temperature that is slightly warmer than the optimum temperature range, around –51°C. Because much of the literature on the effects of freezing and thawing on microorganisms focuses on preservation methods, there has been relatively little study of microorganisms subjected to the slower freezing and thawing rates found in nature. This makes it difficult to develop a model and predict the extent of survival. However, based on the findings from our literature review, including some similar archeological samples, we anticipate that some bacteria, viruses, and helminth ova would survive long-term frozen storage in the Antarctic ice sheet. It is less clear whether the protozoan ova would persist and remain infectious under these conditions, especially *Cryptosporidium* species. Because we anticipate that many species of microorganisms would persist, we recommend taking samples from a series of increasingly aged sewage bulbs in Antarctica and testing them to determine the current viability of these microorganisms.

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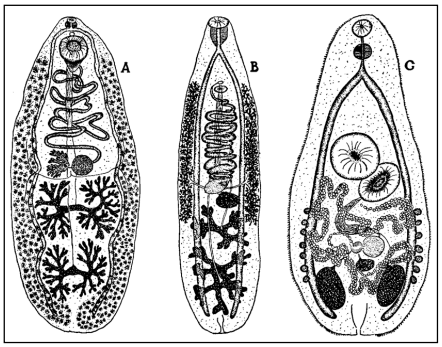
APPENDIX A. HELMINTHS

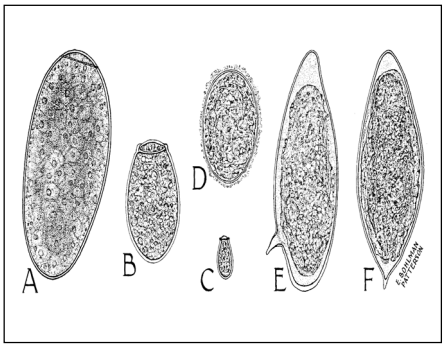
The Platyhelminthes are the helminths of the lowest structural organization. They are flattened and hence the common name flatworm. Unlike nearly all other animals, they have no body cavity; the organisms are embedded in a sort of spongy parenchyma or packing tissue. The simplest digestive systems consist of a blind sac with only a single opening that serves as a mouth and as a vent, although some have an anus. The nervous system is simple and consists of primitive ganglia that serve as a brain and which are located in the anterior portion of the worm. A system of tubes with terminal branches closed, a bush of cilia (that direct the flow of fluid toward the larger branches and ultimately the excretory pore) function as kidneys. The most highly developed organs are those for reproduction, and almost all Platyhelminthes are hermaphroditic. Many of these organisms have special asexual methods of multiplication besides the conventional sexual reproduction. Classes of interest include the Trematoda, which include the flukes, and Cestoidea or tapeworms. Figures A1 and A2 show some of the trematode parasites of man and their eggs, respectively. Figure A3 shows the various life stages of the pork tapeworm *T. solium*.

All the important human parasites in the phylum Nemathelminthes are nematodes. They are elongated, bilaterally symmetrical, unsegmented, cylindrical worms that taper more or less at the head and tail ends. The body cavity is not lined with a peritoneum of mesodermal origin as in higher animals. They have a mouth, an anus, a body cavity (not a true coelom) with a digestive tract that lacks a definite muscular wall, and no respiratory or circulatory system. Male and female reproductive systems usually occur in separate individuals that are noticeably different in general appearance. Life cycles can vary from simple forms of development to those requiring several hosts. They are encased in a very tough, impermeable but flexible transparent or semi-transparent cuticle. This cuticle is not composed of chitin, although the eggs have chitin in the shells. Common nematodes of concern include *Enterobius vermicularis, Trichuris trichiura*, *Ascaris lumbricoides*, and the hookworms *Ancylostoma duodenale* and *Necator americanus*. Figure A4 shows the adult male and female of *Enterobius vermicularis,* and Figure A5 shows the eggs of several nematode species.



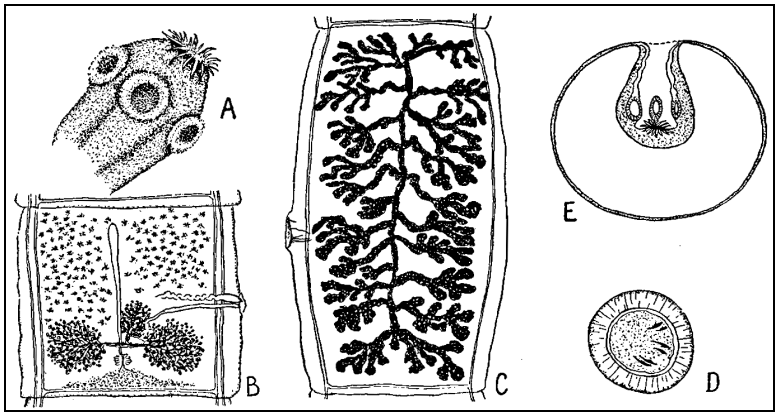
a. *Fasciolopsis buski*.
b. *Clonorchis sinensis*.
c. *Heterophyes heterophyes*.

Figure A1. Trematode parasites of man (at different magnification). (From Lewert 1968.)



a. *Fasciolopsis buski*.
b. *Paragonimus westermani*.
c. *Clonorchis sinensis*.
d. *Schistosoma japonicum*.
e. *Schistosoma mansoni*.
f. *Schistosoma haematobium*.

Figure A2. Trematode eggs. (From Lewert 1968.)



a. Scolex.
b. Mature proglottid.
c. Gravid proglottid.
d. Egg.
e. Cysticercus.

Figure A3. Various life stages of pork tapeworm *Taenia solium* (at different magnification). (From Lewert 1968.)

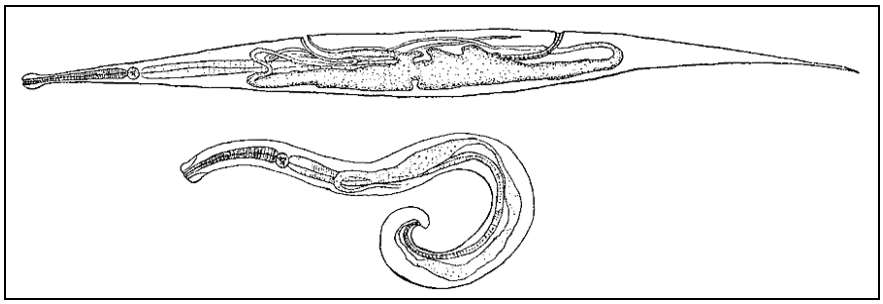
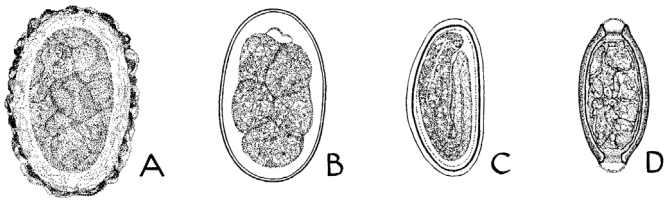


Figure A4. Adult female (top) and male (bottom) of *Enterobius vermicularis*. (From Lewert 1968.)



a. *Ascaris lumbricoides*.

b. *Necator americanus*.

c. *Enterobius vermicularis*.

d. *Trichuris trichiura*.

e. *Strongyloides stercoralis*.

Figure A5. Eggs of several nematode species. (From Lewert 1968.)

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14. ABSTRACT

Microorganisms that are found in domestic wastewater and that can cause illness in humans include bacteria, viruses, protozoan cysts, and helminth ova. This literature review attempts to determine whether organisms contained in a frozen sewage bulb in the Antarctic ice would survive for decades. This review briefly examines the structural differences between these organisms; examines the susceptibility of these organisms to chilling, freezing, thawing, and frozen storage, and the effect these processes have on the structural components of the organisms; and compares findings from field studies, including some archeo-logical studies, on the ability of these organisms to withstand natural cold environments.

15. SUBJECT TERMS

Bacteria	Freezing	Microorganisms	Rapid cooking	Viruses
Cold adaptation	Helminth	Protozoa	Slow cooling	Warming
Cold shock	Helminth eggs	Protozoan cysts	Thawing	Wastewater

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